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Epithelial-mesenchymal transition markers expressed in circulating tumor cells of early and metastatic breast cancer patients

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Introduction: Epithelial-mesenchymal transition (EMT) is considered an essential process in the metastatic cascade. EMT is characterized by up-regulation of Vimentin, Twist, snail, slug and SIP-1 among others. Metastasis is also associated with the presence of circulating (CTCs) and disseminated (DTCs) tumor cells in the blood and bone marrow, respectively, of breast cancer patients, but the expression of EMT markers in these cells has not been reported so far.

Methods: The expression of Twist and Vimentin in CTCs of 25 metastatic and 25 early breast cancer patients was investigated using double immunofluorescence experiments in isolated peripheral blood mononuclear cells (PBMCs)' cytospins using anti-cytokeratin (CK) anti-mouse (A45-B/B3) and anti-Twist or anti-Vimentin anti-rabbit antibodies.

Results: Vimentin- and Twist-expressing CK(+) CTCs were identified in 77% and 73%, respectively, of early breast cancer patients and in 100% for both markers of metastatic patients (P=0.004 and P=0.037, respectively). Among patients with early disease, 56% and 53% of the CK (+) CTCs were double stained with Vimentin and Twist, whereas the corresponding values for metastatic patients were 74% and 97%, respectively (P=0.005 and P=0.0001, respectively). The median expression of CK+Vimentin+ and CK+Twist+ cells per patient in metastatic setting was 98% and 100%, whereas in adjuvant setting the corresponding numbers were 56% and 40.6%, respectively. Triple-staining experiments revealed that all CK^+Twist^+ or $CK^+Vimentin^+$ cells were also CD45-negative, confirming their epithelial origin. Immunomagnetic separation of CTCs and triple immunofluorescence with anti-CK/Twist/Vimentin antibodies demonstrated that both mesenchymal markers could be co-expressed in the same CK(+) cell since 64% of the total identified CTCs were triple stained. There was a significant correlation (P=0.005) between the number of CTCs expressing Twist and Vimentin within the same setting.

Conclusions: CTCs expressing Twist and Vimentin, suggestive of an epithelial-mesenchymal transition, are identified in patients with breast cancer. The high incidence of these cells in patients with metastatic disease compared to early stage breast cancer strongly supports the notion that EMT is involved in the metastatic potential of CTCs.

Introduction

Metastasis is associated with the presence of peripheral blood circulating (CTCs) and bone marrow disseminated (DTCs) tumor cells in patients with breast cancer [1, 2]. In fact the presence of CTCs before the initiation and after the completion of adjuvant chemotherapy is associated with poor clinical outcome [3–5]. In metastatic breast cancer, the assessment of CTCs before and shortly after the initiation of chemotherapy is also predictive of progression-free and overall survival [6, 7] and prognosis seems to depend on the detection of CTCs rather than that of DTCs [8]. The presence of chromosomal alterations confirmed the malignant nature of CTCs [9, 10]. Nevertheless, only some of them are capable of promoting metastasis [11]. Therefore, further molecular characterization of CTCs is essential for understanding their metastatic potential, as well as for the identification of additional markers related to patients' prognosis.

The metastatic process consists of distinct steps including tumor growth, angiogenesis, tumor cell detachment, epithelial to mesenchymal transition (EMT), intravasation, survival within blood and lymphatic vessels and embolization, extravasation, mesenchymal to epithelial transition (MET), formation of micrometastasis and finally growth of macrometastasis [12]. EMT is a process by which epithelial cells loose their epithelial characteristics and acquire a mesenchymal phenotype. EMT increases the metastatic and invasive potential of these cells [13]. Down-regulation of epithelial markers like cytokeratin and E-cadherin, as well as up-regulation of mesenhcymal markers such as Vimentin, N-cadherin and cadherin-11 characterizes the EMT process. Usually, inhibition of E-cadherin expression leads to induction of N-cadherin expression which has been associated with tumor invasiveness [14–16]. Transforming growth factor-β as well as transcription factors such as Twist, Snail, Slug and Sip1 have a regulatory role in EMT program.

Twist is a transcriptional repressor of E-cadherin gene [12, 17]. Increased expression of Twist has been demonstrated in many types of tumor cells like melanoma, osteosarcoma, T cells (Sezary's syndrome), gastric, prostate and breast cancer [12, 18–23]. Gene expression profile of immunomagnetically isolated DTCs showed elevated expression of Twist in the enriched fragment compared to normal volunteers [24, 25]. Twist expression in breast cancer cells has been shown to result in resistance to paclitaxel through binding to Akt promoter and enhancement of its

transcriptional activity [26], as well as resistance to other microtubule targeting agents such as vincristine [27, 28]. Up-regulation of Twist in cancer cells increases VEGF gene expression [21, 29], while HIF-1α regulates the expression of Twist by binding directly to the hypoxia-response element (HRE) in the Twist proximal promoter [30, 31]. We have recently shown that VEGF and HIF-1α, as well as pAkt are expressed in CTCs of most metastatic breast cancer patients [32, 33]; therefore, it was interesting to further investigate the expression of Twist in CTCs of breast cancer patients.

Vimentin is an intermediate filament normally expressed in mesenchymal cells and is involved in the migration of epithelial cells during development [34]. The expression of Vimentin in cancer cells is believed to enhance migration and invasiveness [35]. Expression of Vimentin is characteristic of epithelial cells undergoing EMT process and is related to reduced expression of E-cadherin and up-regulation of N-cadherin [16, 36], while increased expression of Vimentin in breast carcinomas is correlated with poor prognosis [37]. Moreover, the simultaneous expression of Vimentin and cytokeratin in tumor cells is associated with poorer survival in breast cancer patients [38]. Recent studies have demonstrated that Vimentin is expressed in DTCs of breast cancer patients and tumor cell lines [39, 40]; however, there are no studies evaluating the expression of both EMT markers (Twist and Vimentin) in CTCs. Therefore, the aim of the present study was to investigate the expression of these molecules in CTCs of patients with early and metastatic breast cancer.

Materials and methods

Patient samples and cytospin preparation

A longitudinal trial for the study of micrometastatic disease in breast cancer is underway in our Institution since 1996. Peripheral blood samples are obtained from patients who sign an informed consent as part of the routine evaluation, before the initiation and at the end of adjuvant treatment, as well as prior to and after the completion of each chemotherapy line in patients with metastatic disease. Frozen RNA samples and PBMCs' cytospins are prepared simultaneously and stored to -80oC untill their use. In the current trial, samples from patients with adjuvant or metastatic breast cancer were screened for cytokeratin (CK)-19 mRNA expression [41, 42] by RT-PCR. Fifty CK-19 mRNA positive patients (25 with early and 25 with metastatic breast cancer) were enrolled in the present study. We used archived

slides from these patients because we wanted to have the same blood sample for Real Time RT-PCR and immunocytochemistry. Ten healthy female blood donors were also included as a control group. All blood samples were obtained at the middle of vein puncture, after the first 5 ml of blood were discarded. These precautions were undertaken in order to avoid contamination of the blood sample with epithelial cells from the skin during sample collection. All patients and healthy volunteers gave their informed consent to participate in the study, which has been approved by the Ethics and Scientific Committees of our Institution.

The volume of blood from each patient was 20ml both for immunofluorescence and RT-PCR experiments and 20ml for immunomagnetic isolations. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient (d=1,077gr/mol) centrifugation at 1800rpm for 30min. PBMCs were washed three times with PBS and centrifuged at 1500rpm for 10min. Aliquots of 250000 cells were cyto-centrifuged at 2000rpm for 2min on glass slides. Cytospins were dried up and stored at -80^0C. Four to five slides from each patient were used for staining experiments, so 10^6 PBMCs were scanned per patient.

Cell cultures

In control experiments we used the human cervical adenocarcinoma cell line HeLa. HeLa cells express Twist and Vimentin and have been proposed as a positive control by the antibody's data sheet. The HeLa adenocarcinoma cells (obtained from ATCC; American Type Culture Collection) were cultured in (v/v) 1:1 Dulbecco's Modified Eagle Medium (DMEM) (GIBCO-BRL Co, MD, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL Co, MD, USA), 2mM L-glutamine (GIBCO-BRL Co, MD, USA) and 50mg/ml penicilline/streptomycin (GIBCO-BRL Co, MD, USA) Cells were maintained in a humidified atmosphere of 5% CO_2-95% air. Subcultivation for all cell lines was performed using 0.25% trypsin and 5mM EDTA (GIBCO-BRL Co, MD, USA). All experiments were performed during the logarithmic growth phase 15-20 h prior to the experiments. HeLa cells were spiked in blood obtained from healthy volunteers and cytospins were prepared after with Ficoll-Hypaque density gradient centrifugation as per patients' samples.

Double immunofluorescence, Confocal and ARIOL scanning Microscopy

The presence of CK-positive cells in PBMCs' cytospin preparations was investigated using the mouse A45-B/B3 (detecting CK8, CK18 and CK19) (Micromet Munich, Germany) antibody. Control experiments for the sensitivity and the specificity of this antibody have been previously reported [32, 33, 43]. The cytomorphological criteria proposed by Meng et al [44] (i.e. high nuclear/cytoplasmic ratio, larger cells than white blood cells etc) were used in order to characterize a CK-positive cell as a CTC.

Cytospins from the same patients were double stained for Twist/Cytokeratin (Abcam, Cambridge, UK) and Vimentin/Cytokeratin (Santa Cruz, USA) in double staining experiments [45]. Specific staining can be easily distinguished by double immunofluorescence because of the differential intracellular distribution of the examined molecules compared to non-specific staining, as reported by Fehm et al [46]. For double staining experiments PBMC cytospins were fixed with 3% paraformaldehyde (PFA). Permeabilization of the cell membrane was performed with 0.5% Triton for 10min and blocking with PBS/1%BSA overnight. Subsequently, slides were stained for cytokeratin with the A45-B/B3 anti-mouse antibody along with the corresponding secondary (FITC) fluorochrome. Slides were then stained with Twist or Vimentin anti-rabbit antibodies and afterwards with the corresponding anti-rabbit secondary antibodies for 45min. Negative controls were performed for all the primary antibodies, by omitting the corresponding primary antibody and adding the secondary IgG isotype antibody. Finally, Dapi-antifade reagent (Invitrogen Carlsbad, CA, USA) was added to each sample for nuclear staining. Slides were analyzed using a confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany) and ARIOL system (Genetix, UK) CTCs software.

Triple immunofluorescence

Triple immunofluorescence for CK/Twist/CD45, CK/Vimentin/CD45 and CK/Twist/Vimentin was also performed in samples processed by immunomagnetic separation in order to be enriched in CTCs. Cells were initially fixed using 4% formaldehyde for 15min at room temperature (RT). Permeabilization was achieved with 0.1% Triton X-100 for 5min at RT. After blocking with PBS supplemented with 10% (v/v) FBS for 30min, cells were incubated with the corresponding antibodies for 45min each. Zenon technology (FITC-conjugated IGg1 antibody) (Molecular Probes,

Invitrogen, Carlsbad, CA, USA) was used for CK detection with the A45-B/B3 antibody. Zenon antibodies were prepared within 30min before use.

Twist was detected using anti-mouse antibody (Abcam) labelled with Alexa 633 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) or Twist anti-rabbit (Cell Signaling, Boston, USA) labelled with Alexa 555 (Molecular Probes). Positive and negative controls for Twist anti-rabbit are shown in Figure 1A, while positive and negative controls for the Twist anti-mouse antibody are presented in the Additional Figure 1**.** Vimentin was detected using anti-rabbit antibody (Santa Cruz, USA) labelled with Alexa 555; positive and negative controls are shown in Additional Figure 1. CD45 was detected with an anti-mouse antibody (DAKO, Carpinteria, Cal, USA) labelled with Alexa 633. Cells incubated with the different antibodies were post-fixed with 4% (v/v) formaldehyde in PBS for 15min at RT. Finally, cells were stained with DAPI conjugated with antifade.

Immunomagnetic separation of CTCs

A negative selection procedure was used for the isolation of CTCs according to Naume [47]. 100μl of CELLection beads (coated with anti-CD45 monoclonal antibody) (Dynal, Invitrogen) were added in 10^7/ml PBMCs in PBS/0.1% BSA/2mM EDTA. After an incubation of 30min at 4^{o}C the supernatant was transferred in FBS-coated tubes and cells were cytocentrifuged at 2000rpm for 2min on glass slides. The same number of cells was centrifuged at 1500rpm for 5min and the pellet was stored at -80^{o}C for RNA extraction. The specificity and the sensitivity of this method has been previously described [33].

CD45 immunomagnetic depletions were performed for the purpose of triple staining experiments only in order to enrich the samples with CTCs.

Results

Twist and Vimentin expression in HeLa cells and PBMCs of normal blood donors

HeLa cells' cytospins spiked in blood from healthy volunteers and processed as per patients' samples, were used as positive controls for the detection of Twist and Vimentin. Positive and negative controls for Twist, Vimentin and CK are shown in Figure 1.

Twist and Vimentin expression was subsequently investigated in PBMC cytospins of 10 healthy blood donors. Both molecules were found to be expressed spontaneously in PBMCs, but there were no double positive cells (CK^+Twist^+ or $CK^+Vimentin^+$) in healthy volunteers.

Twist and Vimentin expression on CTCs of early and metastatic breast cancer patients

Twenty five patients with metastatic and 25 with early breast cancer, who had detectable CK-19 mRNA-positive cells by RT-PCR were enrolled in this study. The concordance between RT-PCR and immunocytochemistry was 100% in metastatic patients and 88% in early patient. The clinicopathological characteristics of these patients are presented in Table 1.

We investigated the expression of Twist and Vimentin on CTCs using PBMC cytospin preparations from these patients. The presence of CK(+) cells was confirmed by immunofluorescence in all patients with metastatic disease and in 22 out of 25 patients with early disease. Double staining experiments with pancytokeratin and Twist antibodies revealed that all patients with metastatic disease had detectable double stained cells; CK(+)/Twist(+) cells could be detected in 16 (72.7%) out of 22 patients with early breast cancer (p=0.037; Figure 2A I; Table 2). Additionally, the proportion of double-positive CTCs was lower in patients with early than metastatic breast cancer (53% vs 97%, respectively; p=0.0001. Figure 2A II). Confocal laser and ARIOL scanning microscopy revealed that Twist was located both in the cytoplasm and in the nucleus of CTCs (Figure 2B I, 3, 4, Additional Figure 2). The median number of CTCs per patient expressing $Twist^+/CK^+$ was 100% (range 33%-100%) in patients with metastatic disease and 40.6% (range 12%-100%) in patients with early stage breast cancer (Figure 2A III). Nineteen metastatic patients (76%) had only double positive ($Twist^+CK^+$) cells, while in early breast cancer patients only 6 (27%) of the 22 CK-positive patients had exclusively $Twist^+CK^+$ cells (p=0.0001; Table 2).

We subsequently assessed the expression of Vimentin in CTCs of the same cohort of patients. Vimentin was also expressed on CTCs in 100% and 77% of patients with metastatic and early disease, respectively (p=0.004; Figure 2A I; Table 2). Furthermore, the proportion of double-positive CTCs was lower in patients with early compared to metastatic disease (56% vs 74%; p=0.005; Figure 2A II). In addition, the

median proportion of double-positive CTCs per patient with early and metastatic disease was 56% (range, 21,8%-100%) and 98% (range, 16.7%-100%), respectively Figure 2A III). In 12 (48%) out of 25 patients with metastatic disease all the CTCs were double positive (Vimentin$^+$ CK$^+$) compared with only 6 (27%) of 22 patients with early disease who had exclusively Vim$^+$CK$^+$ CTCs (p=0.048; Table 2). Confocal laser and ARIOL scanning microscopy revealed that the intracellular distribution of Vimentin in CTCs was almost identical to that of cytokeratin filaments (Figure 2B II, 3, 4; Additional Figure 2).

A statistically significant correlation (Spearman's rho analysis) was also observed between the number of CTCs expressing Twist and Vimentin in early (p=0.027) and in metastatic (p=0.009) breast cancer patients. This correlation remained significant event when all patients were grouped together (p=0.005).

Triple immunofluorescence experiments revealed specific expression of Twist and Vimentin in CTCs

As mentioned above Twist and Vimentin expression was also observed in PBMCs. In order to confirm that the cells characterized as CTCs in breast cancer patients were non hematopoietic cells presenting ectopic cytokeratin expression, we performed triple immunofluorescence experiments using antibodies against cytokeratins and CD45 along with antibodies against Twist or Vimentin. These experiments were carried out indicatively in five metastatic and eight early breast cancer patients. Our results revealed that no CK$^+$Vimentin$^+$CD45$^+$ or CK$^+$Twist$^+$CD45$^+$ cells could be identified in patients' samples (Figure 3).

Vimentin and Twist are co-expressed in CTCs of breast cancer patients

Subsequently, we investigated the possible co-expression of the examined mesenchymal markers in the same circulating tumor cell. After CD45-negative immunomagnetic separation performed in PBMCs isolated from 24 patients with metastatic disease, the CTC-enriched fraction was cytocentrifuged on one slide for each patient. Triple immunofluorescence experiments using anti- pancytokeratin, Twist and Vimentin antibodies revealed that 16 (64%) out of 25 CTCs identified were CK$^+$Twist$^+$Vimentin$^+$, and 9 (36%) were CK$^+$Twist$^+$Vimentin$^-$. None of the detected CTCs had the CK$^+$Twist$^-$Vimentin$^+$ phenotype. These results indicate that Twist and

Vimentin are co-expressed in a subset of CTCs of metastatic breast cancer patients (Figure 4).

Discussion

There is increasing evidence that the presence of CTCs and DTCs is correlated with minimal residual disease or disease progression in patients with breast cancer. Nevertheless, the underlying molecular characteristics of micrometastatic cells associated with the development of overt metastases remain largely unknown. Epithelial to mesenchymal transition is a multistep process suggested to play a key role in cancer progression and metastasis [12]. Accordingly, CTCs bearing characteristics of an EMT phenotype should be actively involved in tumor dissemination, proliferation and metastasis. Twist is a transcription factor that among others participates in EMT and it is upregulated in many tumor cells [18–22, 48]. In a recent report by Watson et al, Twist expression was specifically enhanced in a gene signature obtained from epCAM-enriched bone marrow samples of patients with breast cancer after neoadjuvant chemotherapy [24]. Twist also increases VEGF expression, while it is directly regulated by HIF-1α [30, 31]. Since we have recently shown that 62% and 76% of breast cancer patients express VEGF and HIF-1α, respectively, in their CTCs [33], it was of interest to identify the potential expression of this transcription factor in CTCs.

In the present cytomorphological study we compared for the first time the expression of Twist and Vimentin in individual CTCs of patients with early vs metastatic breast cancer. For this purpose we performed double staining experiments with the corresponding antibodies in PBMC cytospin preparations of 25 early and 25 metastatic breast cancer patients. In order to confirm the specificity of our results, we also performed triple staining experiments with pancytokeratin/Twist/CD45 and pancytokeratin/Vimentin/CD45 antibodies, which revealed that the hematopoietic antigen CD45 was not expressed in CK/Twist-positive or CK/Vimentin-positive cells; these findings clearly indicate that the observed expression of Twist and Vimentin is not confined to hematopoietic cells.

Double staining experiments revealed that Twist was expressed in all CK-19 mRNA (+) metastatic patients, while most of them (76%) had exclusively double positive cells (CK^+Twist^+); the median expression per patient of Twist in CTCs was also high

100%. Similar results have also been reported in two recent studies by Aktas et al. and by Mego et al [49, 50]. In these studies EMT markers including Twist were detected in CTCs of breast cancer patients at a lower percentage 42%.and 57,7% respectively This lower level of expression might be related to the difference in the identification method or to the fact that immunomagnetic enrichment of tumor cells.

Interestingly, when Vimentin expression was investigated in the same cohort of patients it was observed that, similarly to Twist, Vimentin was expressed in CTCs of all the evaluated patients with metastatic disease and the proportion of patients who had exclusively double positive cells (CK+Vimentin+) was also high. The median percentage of Vimentin-expressing CTCs per patient was 98%. Vimentin is an intermediate filament of mesenchymal cells that is commonly used to identify cells undergoing EMT in cancer. In addition, its expression is associated with increased risk of metastasis and poor prognosis in breast cancer patients [37, 38]. There are only two previous reports verifying the expression of Vimentin in DTCs' cell lines and in CTCs [38, 51]. In agreement with these data, the high rate of Twist and Vimentin expression in CTCs observed in our study implies that mesenchymal markers are highly expressed in CTCs of metastatic breast cancer patients and denotes that the majority of these cells are undergoing EMT process.

Furthermore, our experiments revealed that CK^+Twist^+ and $CK^+Vimentin^+$ CTCs were also observed in patients with early stage breast cancer. This observation suggests that circulating epithelial (CK+) cells with an EMT phenotype may be involved in the continuous tumor spreading in patients with clinically undetectable metastases. However, the EMT phenotype expression was significantly lower ($p=0.037$ for Twist and $p=0.004$ for Vimentin) in early compared to metastatic patients. The median expression of Twist and Vimentin was also significantly lower 40.6% and 55.6%, but extremely variable from patient to patient (range, 11%-100% and 21%-100%, respectively). The proportion of double positive CTCs was 53% and 56% for Twist and Vimentin, respectively, which was also significantly different from metastatic setting ($p=0.0001$ and $p=0.05$). These findings clearly indicate that EMT markers may be expressed in CTCs of patients with early disease but to a lesser extent than in patients with metastatic disease. In addition, the above results imply that the population of CTCs expressing EMT markers predominates during disease progression or alternatively, it is selected over non-EMT expressing CTCs due to resistance to therapy. However, this finding should be interpreted with caution,

because of the smaller number of CTCs detected in patients with early compared to advanced disease, which might influence the chance to find an EMT-like CTC.

A statistically significant correlation between the number of CTCs expressing Twist and Vimentin both in patients with early and metastatic disease was demonstrated ($p=0.005$ and $p=0.027$), suggesting that both markers were simultaneously expressed in CTCs. This assumption was confirmed by triple staining experiments using antibodies against CK, Twist and Vimentin in immunomagnetically isolated CTCs from 24 patients, which demonstrated that the majority (64%) of CTCs had the $CK^{+}Twist^{+}Vimentin^{+}$ phenotype. Nevertheless, 36% of the CTCs were $CK^{+}Twist^{+}Vimentin^{-}$, whereas there were no cells with the CK+Twist-Vimentin+ phenotype (Figure 4). These findings indicate the heterogeneity of CTCs. It is also possible that Twist expression is a more common phenomenon during EMT, thus suggesting Twist as a more specific marker for EMT.

Finally, a differential intracellular distribution for Vimentin and Twist was observed in CTCs. Vimentin was primarily located in the cytoplasm, similarly to the intracellular distribution of cytokeratin (Figure 2, 3, 4). This observation is in agreement with previous reports showing that Vimentin filaments follow the pre-existing cytokeratin network during EMT progression [52]. Twist showed both cytoplasmic and nuclear localization as expected considering its function as a transcription factor (Figure 2, 3, 4).

Conclusions

The results of the present study clearly indicate that EMT markers such as Twist and Vimentin are expressed in CTCs of patients with early and metastatic breast cancer. The variable expression of these molecules in CTCs with different stages of disease, imply the predominance of EMT phenotype during disease evolution. This hypothesis is further supported by the observation that CK(+) CTCs in patients with early breast cancer are more heterogeneous concerning the expression of EMT markers, necessitating additional studies to further elucidate their distinct biological role.

Abbreviations

ARIOL system: automated Image Analysis System; CTCs: Circulating Tumor Cells; DTCs: Disseminated Tumor Cells; ECOG: Eastern Cooperative Oncology Group; EMT: Epithelial-Mesenchymal Transition; FITC: fluorescein isothiocyanate; HeLa: patient with cervical adenocarcinoma from which the cell line was derived; PBMCs: Peripheral Blood Mononuclear Cells.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GK participated in the design and coordination of the study. She performed the immunomagnetic separations and the cell cultures. She also drafted the manuscript. MP and EP performed the immunofluorescence experiments and involved in drafting the manuscript. SA helped drafted the manuscript. She also collected all the clinicopathological characteristics of the patients. DM helped to draft the manuscript and participated in study design, VG provided general support, participated in study design and involved in drafting the manuscript. All the authors have given final approval of the version to be published.

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References

1. Braun S , Naume B: **Circulating and disseminated tumor cells**. *J Clin Oncol* 2005, **23:** 1623-1626.
2. Pantel K, Muller V, Auer M, Nusser N, Harbeck N, Braun S: **Detection and clinical implications of early systemic tumor cell dissemination in breast cancer**. *Clin Cancer Res* 2003, **9:** 6326-6334.
3. Lobodasch K, Frohlich F, Rengsberger M, Schubert R, Dengler R, Pachmann U, Pachmann K: **Quantification of circulating tumour cells for the monitoring of adjuvant therapy in breast cancer: an increase in cell number at completion of therapy is a predictor of early relapse**. *Breast* 2007, **16:** 211-218.
4. Stathopoulou A, Vlachonikolis I, Mavroudis D, Perraki M, Kouroussis C, Apostolaki S, Malamos N, Kakolyris S, Kotsakis A, Xenidis N, Reppa D, Georgoulias V: **Molecular detection of cytokeratin-19-positive cells in the peripheral blood of patients with operable breast cancer: evaluation of their prognostic significance**. *J Clin Oncol* 2002, **20:** 3404-3412.
5. Xenidis N, Perraki M, Kafousi M, Apostolaki S, Bolonaki I, Stathopoulou A, Kalbakis K, Androulakis N, Kouroussis C, Pallis T, Christophylakis C, Argyraki K, Lianidou ES, Stathopoulos S, Georgoulias V, Mavroudis D: **Predictive and prognostic value of peripheral blood cytokeratin-19 mRNA-positive cells detected by real-time polymerase chain reaction in node-negative breast cancer patients**. *J Clin Oncol* 2006, **24:** 3756-3762.
6. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW, Hayes DF: **Circulating tumor**

cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 2004, **351:** 781-791.

7. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, Doyle GV, Matera J, Allard WJ, Miller MC, Fritsche HA, Hortobagyi GN, Terstappen LW: **Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer**. *J Clin Oncol* 2005, **23:** 1420-1430.
8. Bidard FC, Vincent-Salomon A, Sigal-Zafrani B, Dieras V, Mathiot C, Mignot L, Thiery JP, Sastre-Garau X, Pierga JY: **Prognosis of women with stage IV breast cancer depends on detection of circulating tumor cells rather than disseminated tumor cells**. *Ann Oncol* 2008, **19:** 496-500.
9. Bozionellou V, Mavroudis D, Perraki M, Papadopoulos S, Apostolaki S, Stathopoulos E, Stathopoulou A, Lianidou E, Georgoulias V: **Trastuzumab administration can effectively target chemotherapy-resistant cytokeratin-19 messenger RNA-positive tumor cells in the peripheral blood and bone marrow of patients with breast cancer**. *Clin Cancer Res* 2004, **10:** 8185-8194.
10. Fehm T, Sagalowsky A, Clifford E, Beitsch P, Saboorian H, Euhus D, Meng S, Morrison L, Tucker T, Lane N, Ghadimi BM, Heselmeyer-Haddad K, Ried T, Rao C, Uhr J: **Cytogenetic evidence that circulating epithelial cells in patients with carcinoma are malignant**. *Clin Cancer Res* 2002, **8:** 2073-2084.
11. Klein CA, Blankenstein TJ, Schmidt-Kittler O, Petronio M, Polzer B, Stoecklein NH, Riethmuller G: **Genetic heterogeneity of single disseminated tumour cells in minimal residual cancer**. *Lancet* 2002, **360:** 683-689.

12. Paterlini-Brechot P , Benali NL: **Circulating tumor cells (CTC) detection: clinical impact and future directions**. *Cancer Lett* 2007, **253:** 180-204.

13. Guarino M: **Epithelial-mesenchymal transition and tumour invasion**. *Int J Biochem Cell Biol* 2007, **39:** 2153-2160.

14. Behrens J: **Cadherins and catenins: role in signal transduction and tumor progression**. *Cancer Metastasis Rev* 1999, **18:** 15-30.

15. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA: **The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression**. *Nat Cell Biol* 2000, **2:** 76-83.

16. Nakajima S, Doi R, Toyoda E, Tsuji S, Wada M, Koizumi M, Tulachan SS, Ito D, Kami K, Mori T, Kawaguchi Y, Fujimoto K, Hosotani R, Imamura M: **N-cadherin expression and epithelial-mesenchymal transition in pancreatic carcinoma**. *Clin Cancer Res* 2004, **10:** 4125-4133.

17. Vesuna F, Van DP, Chen JH, Raman V: **Twist is a transcriptional repressor of E-cadherin gene expression in breast cancer**. *Biochem Biophys Res Commun* 2008, **367:** 235-241.

18. Entz-Werle N, Stoetzel C, Berard-Marec P, Kalifa C, Brugiere L, Pacquement H, Schmitt C, Tabone MD, Gentet JC, Quillet R, Oudet P, Lutz P, Babin-Boilletot A, Gaub MP, Perrin-Schmitt F: **Frequent genomic abnormalities at TWIST in human pediatric osteosarcomas**. *Int J Cancer* 2005, **117:** 349-355.

19. Hoek K, Rimm DL, Williams KR, Zhao H, Ariyan S, Lin A, Kluger HM, Berger AJ, Cheng E, Trombetta ES, Wu T, Niinobe M, Yoshikawa K, Hannigan GE, Halaban R: **Expression profiling reveals novel pathways in**

the transformation of melanocytes to melanomas. *Cancer Res* 2004, **64:** 5270-5282.

20. Kwok WK, Ling MT, Lee TW, Lau TC, Zhou C, Zhang X, Chua CW, Chan KW, Chan FL, Glackin C, Wong YC, Wang X: **Up-regulation of TWIST in prostate cancer and its implication as a therapeutic target**. *Cancer Res* 2005, **65:** 5153-5162.
21. Rosivatz E, Becker I, Specht K, Fricke E, Luber B, Busch R, Hofler H, Becker KF: **Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer**. *Am J Pathol* 2002, **161:** 1881-1891.
22. van DR, Dijkman R, Vermeer MH, Out-Luiting JJ, van der Raaij-Helmer EM, Willemze R, Tensen CP: **Aberrant expression of the tyrosine kinase receptor EphA4 and the transcription factor twist in Sezary syndrome identified by gene expression analysis**. *Cancer Res* 2004, **64:** 5578-5586.
23. Watanabe O, Imamura H, Shimizu T, Kinoshita J, Okabe T, Hirano A, Yoshimatsu K, Konno S, Aiba M, Ogawa K: **Expression of twist and wnt in human breast cancer**. *Anticancer Res* 2004, **24:** 3851-3856.
24. Watson MA, Ylagan LR, Trinkaus KM, Gillanders WE, Naughton MJ, Weilbaecher KN, Fleming TP, Aft RL: **Isolation and molecular profiling of bone marrow micrometastases identifies TWIST1 as a marker of early tumor relapse in breast cancer patients**. *Clin Cancer Res* 2007, **13:** 5001-5009.
25. Bonnomet A, Brysse A, Tachsidis A, Waltham M, Thompson EW, Polette M, Gilles C: **Epithelial-to-mesenchymal transitions and circulating tumor cells**. *J Mammary Gland Biol Neoplasia* 2010, **15:** 261-273.

26. Cheng GZ, Chan J, Wang Q, Zhang W, Sun CD, Wang LH: **Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel**. *Cancer Res* 2007, **67:** 1979-1987.

27. Wang X, Ling MT, Guan XY, Tsao SW, Cheung HW, Lee DT, Wong YC: **Identification of a novel function of TWIST, a bHLH protein, in the development of acquired taxol resistance in human cancer cells**. *Oncogene* 2004, **23:** 474-482.

28. Zhang X, Wang Q, Ling MT, Wong YC, Leung SC, Wang X: **Anti-apoptotic role of TWIST and its association with Akt pathway in mediating taxol resistance in nasopharyngeal carcinoma cells**. *Int J Cancer* 2007, **120:** 1891-1898.

29. Mironchik Y, Winnard PT, Jr., Vesuna F, Kato Y, Wildes F, Pathak AP, Kominsky S, Artemov D, Bhujwalla Z, Van DP, Burger H, Glackin C, Raman V: **Twist overexpression induces in vivo angiogenesis and correlates with chromosomal instability in breast cancer**. *Cancer Res* 2005, **65:** 10801-10809.

30. Yang MH , Wu KJ: **TWIST activation by hypoxia inducible factor-1 (HIF-1): implications in metastasis and development**. *Cell Cycle* 2008, **7:** 2090-2096.

31. Yang MH, Wu MZ, Chiou SH, Chen PM, Chang SY, Liu CJ, Teng SC, Wu KJ: **Direct regulation of TWIST by HIF-1alpha promotes metastasis**. *Nat Cell Biol* 2008, **10:** 295-305.

32. Kallergi G, Agelaki S, Kalykaki A, Stournaras C, Mavroudis D, Georgoulias V: **Phosphorylated EGFR and PI3K/Akt signaling kinases are expressed**

in circulating tumor cells of breast cancer patients. *Breast Cancer Res* 2008, **10:** R80.

33. Kallergi G, Markomanolaki H, Giannoukaraki V, Papadaki MA, Strati A, Lianidou ES, Georgoulias V, Mavroudis D, Agelaki S: **Hypoxia-inducible factor-1alpha and vascular endothelial growth factor expression in circulating tumor cells of breast cancer patients**. *Breast Cancer Res* 2009, **11:** R84.
34. Franke WW, Grund C, Kuhn C, Jackson BW, Illmensee K: **Formation of cytoskeletal elements during mouse embryogenesis. III. Primary mesenchymal cells and the first appearance of vimentin filaments**. *Differentiation* 1982, **23:** 43-59.
35. McInroy L , Maatta A: **Down-regulation of vimentin expression inhibits carcinoma cell migration and adhesion**. *Biochem Biophys Res Commun* 2007, **360:** 109-114.
36. Zhao Y, Yan Q, Long X, Chen X, Wang Y: **Vimentin affects the mobility and invasiveness of prostate cancer cells**. *Cell Biochem Funct* 2008, **26:** 571-577.
37. Domagala W, Lasota J, Bartkowiak J, Weber K, Osborn M: **Vimentin is preferentially expressed in human breast carcinomas with low estrogen receptor and high Ki-67 growth fraction**. *Am J Pathol* 1990, **136:** 219-227.
38. Thomas PA, Kirschmann DA, Cerhan JR, Folberg R, Seftor EA, Sellers TA, Hendrix MJ: **Association between keratin and vimentin expression, malignant phenotype, and survival in postmenopausal breast cancer patients**. *Clin Cancer Res* 1999, **5:** 2698-2703.

39. Willipinski-Stapelfeldt B, Riethdorf S, Assmann V, Woelfle U, Rau T, Sauter G, Heukeshoven J, Pantel K: **Changes in cytoskeletal protein composition indicative of an epithelial-mesenchymal transition in human micrometastatic and primary breast carcinoma cells**. *Clin Cancer Res* 2005, **11:** 8006-8014.

40. Bartkowiak K, Wieczorek M, Buck F, Harder S, Moldenhauer J, Effenberger KE, Pantel K, Peter-Katalinic J, Brandt BH: **Two-dimensional differential gel electrophoresis of a cell line derived from a breast cancer micrometastasis revealed a stem/ progenitor cell protein profile**. *J Proteome Res* 2009, **8:** 2004-2014.

41. Ring A, Smith IE, Dowsett M: **Circulating tumour cells in breast cancer**. *Lancet Oncol* 2004, **5:** 79-88.

42. Stathopoulou A, Gizi A, Perraki M, Apostolaki S, Malamos N, Mavroudis D, Georgoulias V, Lianidou ES: **Real-time quantification of CK-19 mRNA-positive cells in peripheral blood of breast cancer patients using the lightcycler system**. *Clin Cancer Res* 2003, **9:** 5145-5151.

43. Kallergi G, Mavroudis D, Georgoulias V, Stournaras C: **Phosphorylation of FAK, PI-3K, and impaired actin organization in CK-positive micrometastatic breast cancer cells**. *Mol Med* 2007, **13:** 79-88.

44. Meng S, Tripathy D, Frenkel EP, Shete S, Naftalis EZ, Huth JF, Beitsch PD, Leitch M, Hoover S, Euhus D, Haley B, Morrison L, Fleming TP, Herlyn D, Terstappen LW, Fehm T, Tucker TF, Lane N, Wang J, Uhr JW: **Circulating tumor cells in patients with breast cancer dormancy**. *Clin Cancer Res* 2004, **10:** 8152-8162.

45. Kallergi G, Mavroudis D, Georgoulias V, Stournaras C: **Phosphorylation of FAK, PI-3K, and impaired actin organization in CK-positive micrometastatic breast cancer cells**. *Mol Med* 2007, **13:** 79-88.

46. Fehm T, Solomayer EF, Meng S, Tucker T, Lane N, Wang J, Gebauer G: **Methods for isolating circulating epithelial cells and criteria for their classification as carcinoma cells**. *Cytotherapy* 2005, **7:** 171-185.

47. Naume B, Borgen E, Nesland JM, Beiske K, Gilen E, Renolen A, Ravnas G, Qvist H, Karesen R, Kvalheim G: **Increased sensitivity for detection of micrometastases in bone-marrow/peripheral-blood stem-cell products from breast-cancer patients by negative immunomagnetic separation**. *Int J Cancer* 1998, **78:** 556-560.

48. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA: **Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis**. *Cell* 2004, **117:** 927-939.

49. Aktas B, Tewes M, Fehm T, Hauch S, Kimmig R, Kasimir-Bauer S: **Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients**. *Breast Cancer Res* 2009, **11:** R46.

50. Mego M, Mani SA, Lee BN, Li C, Evans KW, Cohen EN, Gao H, Jackson SA, Giordano A, Hortobagyi GN, Cristofanilli M, Lucci A, Reuben JM: **Expression of epithelial-mesenchymal transition-inducing transcription factors in primary breast cancer: The effect of neoadjuvant therapy**. *Int J Cancer* 2011, [Epub ahead of print].

51. Raimondi C: **Epithelial-mesenchymal transition and stemness features in circulating tumor cells from breast cancer patients**. Breast Cancer Res Treat 2011, [Epub ahead of print].

52. Pagan R, Martin I, Alonso A, Llobera M, Vilaro S: **Vimentin filaments follow the preexisting cytokeratin network during epithelial-mesenchymal transition of cultured neonatal rat hepatocytes**. *Exp Cell Res* 1996, **222:** 333-344.

Figure legends

Figure 1: Twist and Vimentin expression in HeLa cells spiked in blood of normal volunteers

ARIOL system images of HeLa cells spiked in the blood of normal volunteers.

A. (I) Positive control for Twist; HeLa cells were stained with pancytokeratin A45-B/B3 antibody/secondary FITC anti-mouse antibody (green)/Twist anti-rabbit antibody/Alexa555 anti-rabbit antibody (orange). (II) Negative control for Twist; cells were stained with pancytokeratin A45-B/B3 antibody/FITC anti-mouse (green) and Alexa555 IgG isotype antibody. Cells' nuclei were stained with Dapi (blue). Magnification: (x400).

B. (I) Positive control for Vimentin; cells were stained with pancytokeratin A45-B/B3 antibody/FITC anti-mouse antibody (green) /Vimentin anti-rabbit antibody /Alexa555 anti-rabbit antibody (orange). (II) Negative control for Vimentin; cells were stained with pancytokeratin A45-B/B3 antibody/FITC anti-mouse antibody (green) and Alexa555 IGg isotype antibody. (III) Negative control for pancytokeratin; cells were stained with FITC IgG isotype antibody/Vimentin anti-rabbit antibody /Alexa555 anti-rabbit antibody (orange). Cells' nuclei were stained with Dapi (blue). Magnification: (x400).

CK: cytokeratin; FITC: fluorescein isothiocyanate; HeLa: patient with cervical adenocarcinoma from which the cell line was derived.

Figure 2: Twist and Vimentin expression in CTCs of early and metastatic breast cancer patients

A: (I) Quantification of 25 early and 25 metastatic breast cancer patients harvesting double positive cells of each examined molecule. (II) Quantification of double positive CTCs/Total CTCs for each examined molecule. (III) Quantification of median expression per patient for each examined molecule.

B: Representative ARIOL system images of CTCs in PBMCs' cytospin.

(I): Cytospin double stained with monoclonal pancytokeratin A45-B/B3 (green)/polyclonal Twist anti-rabbit (red) antibodies and Dapi nuclear staining. Magnification: (x400)

(II): Cytospin double stained with monoclonal A45-B/B3 (green)/polyclonal Vimentin anti-rabbit (red) antibodies and Dapi nuclear staining. Magnification: (x400)

CK: cytokeratin; CTCs: circulating tumor cells; PBMCs: Peripheral Blood Mononuclear Cells.

Figure 3: Triple immunofluorescence (CK/Twist/CD45, CK/Vimentin/CD45) in CTCs

Representative ARIOL system micrographs of CTCs' cytospin after negative immunomagnetic separation in patients with metastatic breast cancer.

A: Cells were triple stained with pancytokeratin A45-B/B3 antibody/Zenon-Alexa488 (green)/ Twist anti-rabbit antibody/Alexa555 anti-rabbit antibody (orange) and CD45 anti-mouse/Alexa633 anti mouse antibody (blue). Magnification: (x400)

B: Cells were triple stained with pancytokeratin A45-B/B3 antibody/Zenon-Alexa488 (green)/Vimentin anti-rabbit antibody/Alexa555 anti-rabbit antibody (orange) and CD45 anti-mouse/Alexa633 anti-mouse antibody (blue). Magnification: (x400)

ARIOL system: automated image analysis system; CK: cytokeratin; CTCs: circulating tumor cells.

Figure 4: Co-expression of CK, Twist and Vimentin in the same cell

Representative confocal laser scanning micrographs of CTCs' cytospin after negative immunomagnetic separation in patient with metastatic breast cancer. Cells were triple stained with pancytokeratin A45-B/B3 antibody/Zenon-Alexa488 (green)/Twist anti-mouse/Alexa633 anti-mouse antibody/Vimentin anti-rabbit/Alexa555 anti-rabbit antibody (orange). (Magnification x600)

A: A CTC expressing CK, Twist and Vimentin.

B: A CTC expressing CK and Twist, but not Vimentin.

CK: cytokeratin; CTCs: circulating tumor cells.

Additional files

Additional Figure 1: Triple staining control experiments in HeLa cells

Triple staining control experiments in HeLa cells analyzed with a confocal laser scanning microscope.

(I): Representative confocal laser scanning micrographs of positive controls for Twist anti-mouse antibody; HeLa cells were stained with pancytokeratin A45-B/B3 antibody/Zenon-Alexa488 (green)/Twist anti-mouse/Alexa 633 anti-mouse IGg (blue)/Vimentin anti-rabbit antibody/Alexa555 anti-rabbit antibody (red). Magnification: (x500).

(II): Negative control for Twist; HeLa cells stained with pancytokeratin A45-B/B3 antibody/Zenon-Alexa488 (green)/Alexa 633 anti-mouse IGg (blue)/Vimentin anti-rabbit antibody/ Alexa555 anti-rabbit antibody (red). Magnification: (x500).

(II): Negative control for Vimentin; HeLa cells were stained with pancytokeratin A45-B/B3 antibody/Zenon-Alexa488 (green)/ Twist anti-mouse antibody/Alexa 633 anti-mouse antibody (blue)/Alexa 555 IgG isotype antibody (red). Magnification: (x500).

CK: cytokeratin; HeLa: patient with cervical adenocarcinoma, from which the cell line was derived.

Additional Figure 2: Twist and Vimentin expression in CTCs of breast cancer patients

Representative confocal laser scanning images of Twist- or Vimentin-expressing CTCs of breast cancer patients.

(I): Representative confocal laser scanning images of breast cancer patients' cytospin double stained with monoclonal pancytokeratin (A45-B/B3) /FITC 488 anti-mouse antibody (green)/polyclonal Twist anti-rabbit antibody and Alexa 555 anti-rabbit antibody (red). Magnification: (x600). (II): Representative images of cytospin double stained with monoclonal pancytokeratin (A45-B/B3)/FITC 488 anti-mouse antibody (green)/polyclonal Vimentin anti-rabbit antibody and Alexa 555 anti-rabbit antibody (red). Magnification: (x600).

CK: cytokeratin; CTCs: circulating tumor cells.

Table 1: Patient characteristics

Early Breast Cancer	Metastatic Breast Cancer
No. of patients enrolled 25	No. of patients enrolled 25
Age, years	**Age, years**
Median, range 59 (26-76)	Median, range 59 (36 – 83)
ECOG performance status	**ECOG performance status**
0 -> 24 (96)	0 -> 8 (32)
1 -> 1 (4)	1 -> 13 (52)
2 -	2 -> 4 (16)
Histology	**Histology**
Ductal -> 22 (88)	Ductal -> 20 (80)
Lobular -> 3 (12)	Lobular -> 1 (4)
Other -	UN -> 4 (16)
Menopausal status	**Menopausal status**
Premenopausal -> 8 (32)	Premenopausal -> 4 (16)
Perimenopausal -> 1 (4)	Perimenopausal -> 1 (4)
Postmenopausal -> 16 (64)	Postmenopausal -> 20 (80)
Hormone receptor status	**Hormone receptor status**
ER positive/PR positive -> 11 (44)	ER positive/PR positive -> 11 (44)
ER positive/PR negative -> 6 (24)	ER positive/PR negative -> 3 (12)
ER negative/PR positive -> 1 (4)	ER negative/PR positive -> 2 (8)
ER negative/PR negative -> 7 (28)	ER negative/PR negative -> 9 (36)
Tumor size	**No of Disease sites**

1-1.9 cm (T1) -> 6 (24)

2-5 cm (T2) -> 16 (64)

>5 cm (T3) -> 1 (4)

UN -> 2 (8)

Grade

I -> 2 (8)

II -> 9 (36)

III -> 13 (52)

UN -> 1 (4)

Positive nodes

0 (N0) -> 11 (44)

1-3 (N1) -> 9 (36)

4-9 (N2) -> 2 (8)

≥ 10 (N3) -> 2 (8)

1 -> 7 (28)

2 -> 9 (36)

3 -> 4 (16)

≥4 -> 5 (20)

Line of treatment

1st -> 11 (44)

2nd -> 7 (28)

≥3rd -> 7 (28)

Primary breast cancer at presentation

Early -> 9 (36), Metastatic -> 16 (64)

Visceral disease

Yes -> 13 (52)

No -> 12 (48)

ECOG: Eastern Cooperative Oncology Group; ER: estrogen receptor; PR, progesterone receptor.

Table 2: Number of double stained CTCs/250000 PBMCs in early and metastatic patients

	Early breast cancer patient					Metastatic			
Patient	**Vim^+ CK^+**	**Vim^- CK^+**	**$Twist^+CK^+$**	**$Twist^-CK^+$**	Patient	**Vim^+ CK^+**	**Vim^- CK^+**	**$Twist^+CK^+$**	**$Twist^-CK^+$**
1	64	51	83	100	1	5	0	4	0
2	25	63	29	142	2	45	0	8	0
3	31	111	578	68	3	21	0	7	0
4	35	28	53	85	4	2	0	4	0
5	135	199	18	134	5	74	20	45	0
6	100	158	28	170	6	7	7	3	6
7	170	254	89	423	7	7	2	420	0
8	0	0	74	4	8	21	69	2	0
9	0	1	71	95	9	10	90	3	0
10	140	4	0	1	10	2	0	2	0
11	22	0	0	0	11	3	15	103	36
12	17	0	74	88	12	67	3	9	1
13	0	4	8	0	13	4	0	0	0
14	0	0	0	3	14	3	3	42	0
15	7	1	0	4	15	1	0	1	0
16	10	3	12	0	16	1	0	1	0
17	0	0	0	0	17	10	2	55	0
18	28	12	0	0	18	325	97	5	7
19	0	0	0	10	19	17	1	13	0
20	0	1	2	0	20	3	0	0	0
21	2	0	13	0	21	2	0	2	0
22	2	0	0	9	22	2	1	2	0
23	306	0	380	0	23	9	0	658	0
24	1	0	0	2	24	192	4	44	0
25	24	0	1	0	25	40	0	80	0

Vim: vimentin; CK: cytokeratin; PBMCs: Peripheral Blood Mononuclear Cells.

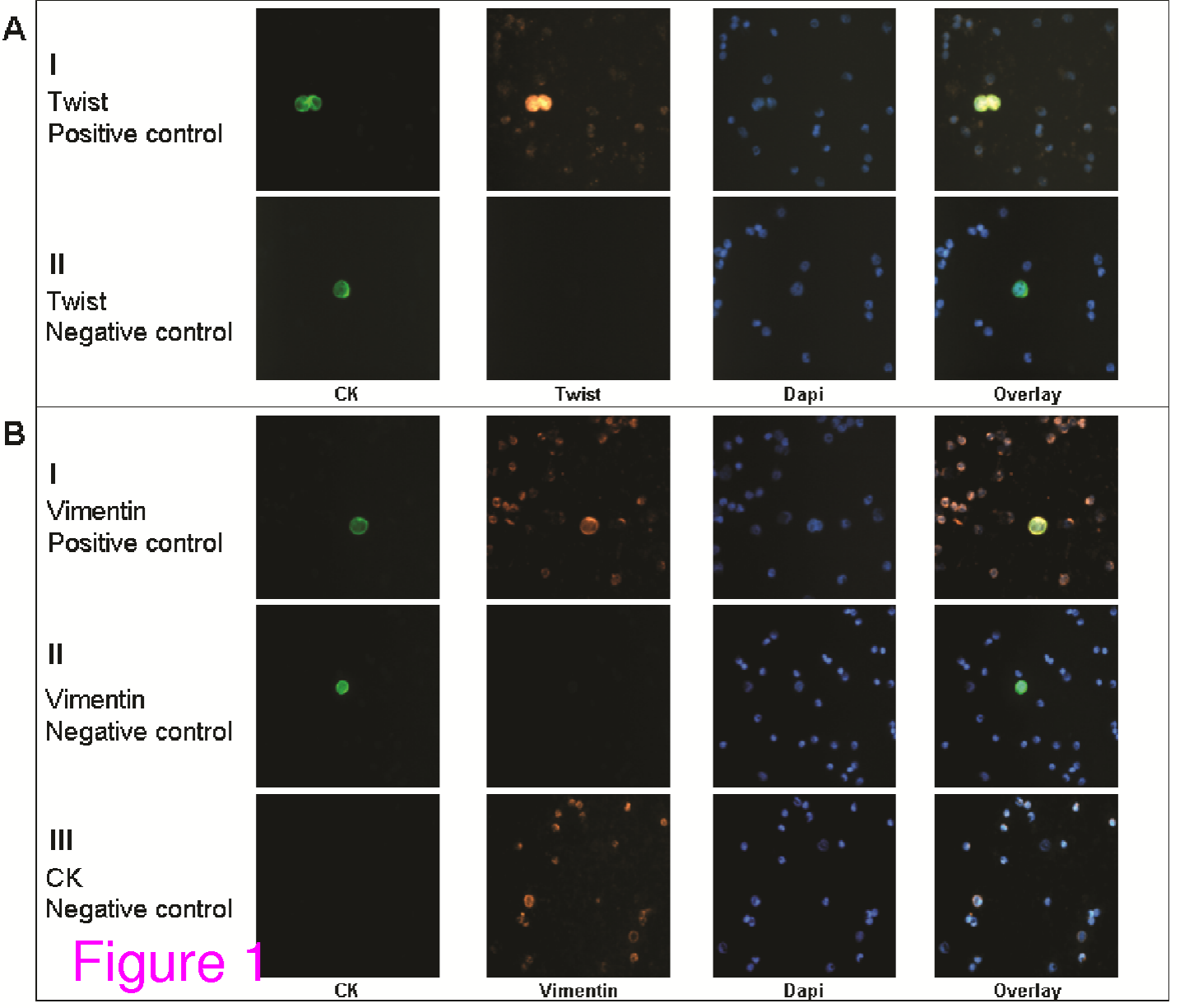
A
I
Twist
Positive control
II
Twist
Negative control
CK
Twist
Dapi
Overlay
B
I
Vimentin
Positive control
II
Vimentin
Negative control
III
CK
Negative control
CK
Vimentin
Dapi
Overlay

Figure 1

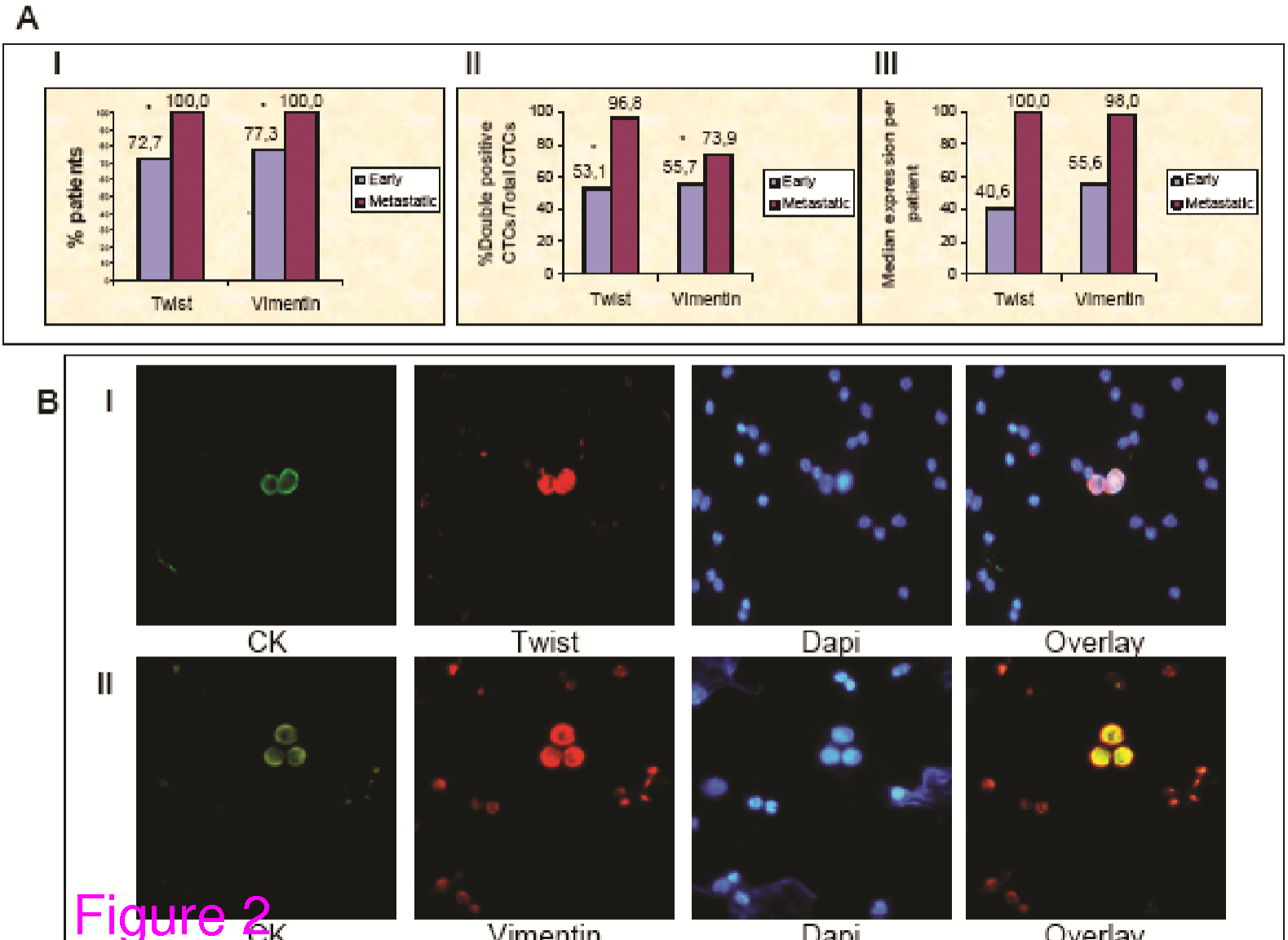
A
I
% patients
72,7
100,0
77,3
100,0
Twist
Vimentin
Early
Metastatic
II
%Double positive CTCs/Total CTCs
53,1
96,8
55,7
73,9
Twist
Vimentin
Early
Metastatic
III
Median expression per patient
40,6
100,0
55,6
98,0
Twist
Vimentin
Early
Metastatic
B
I
CK
Twist
Dapi
Overlay
II
CK
Vimentin
Dapi
Overlay

Figure 2

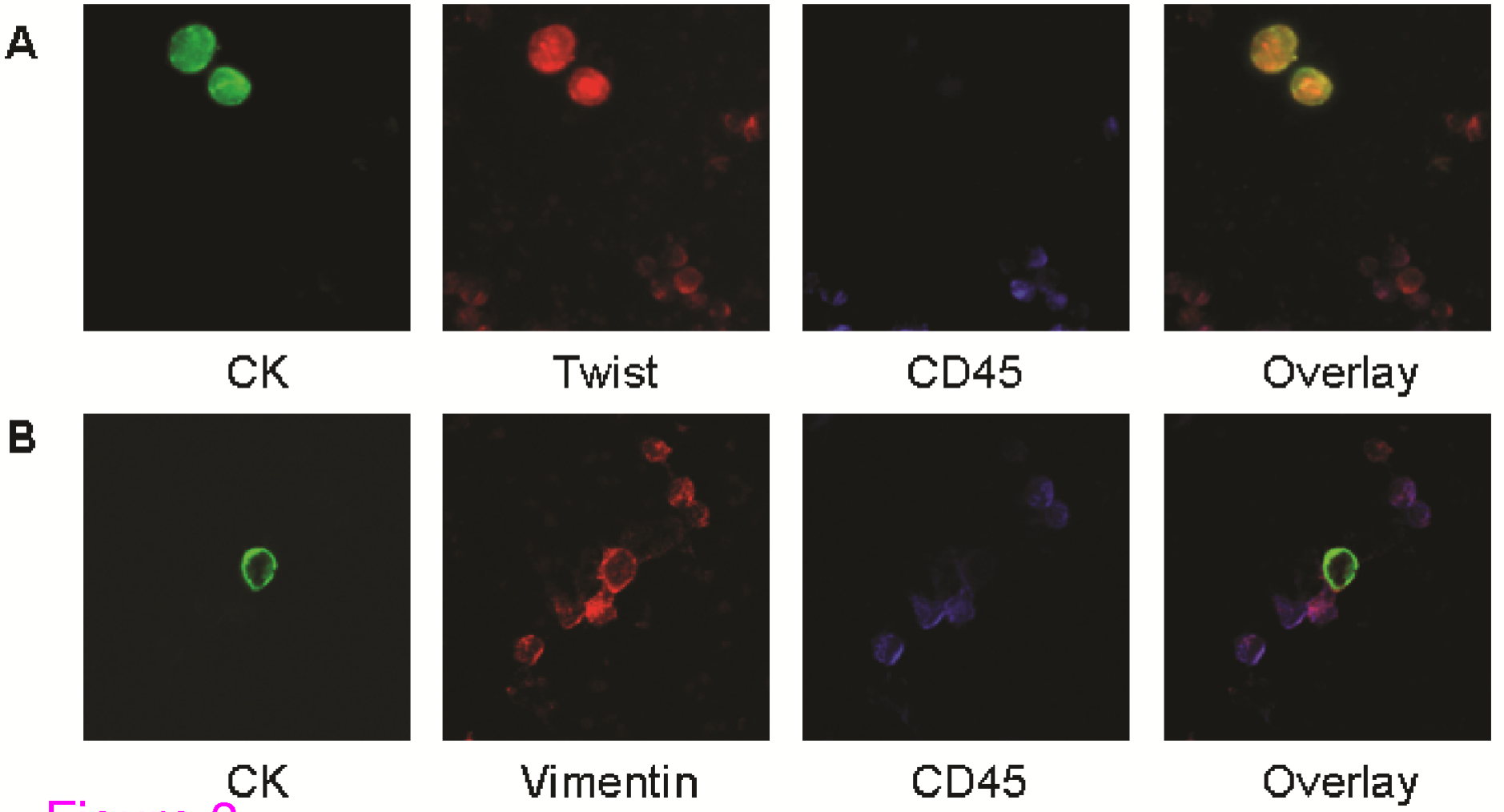

Figure 3

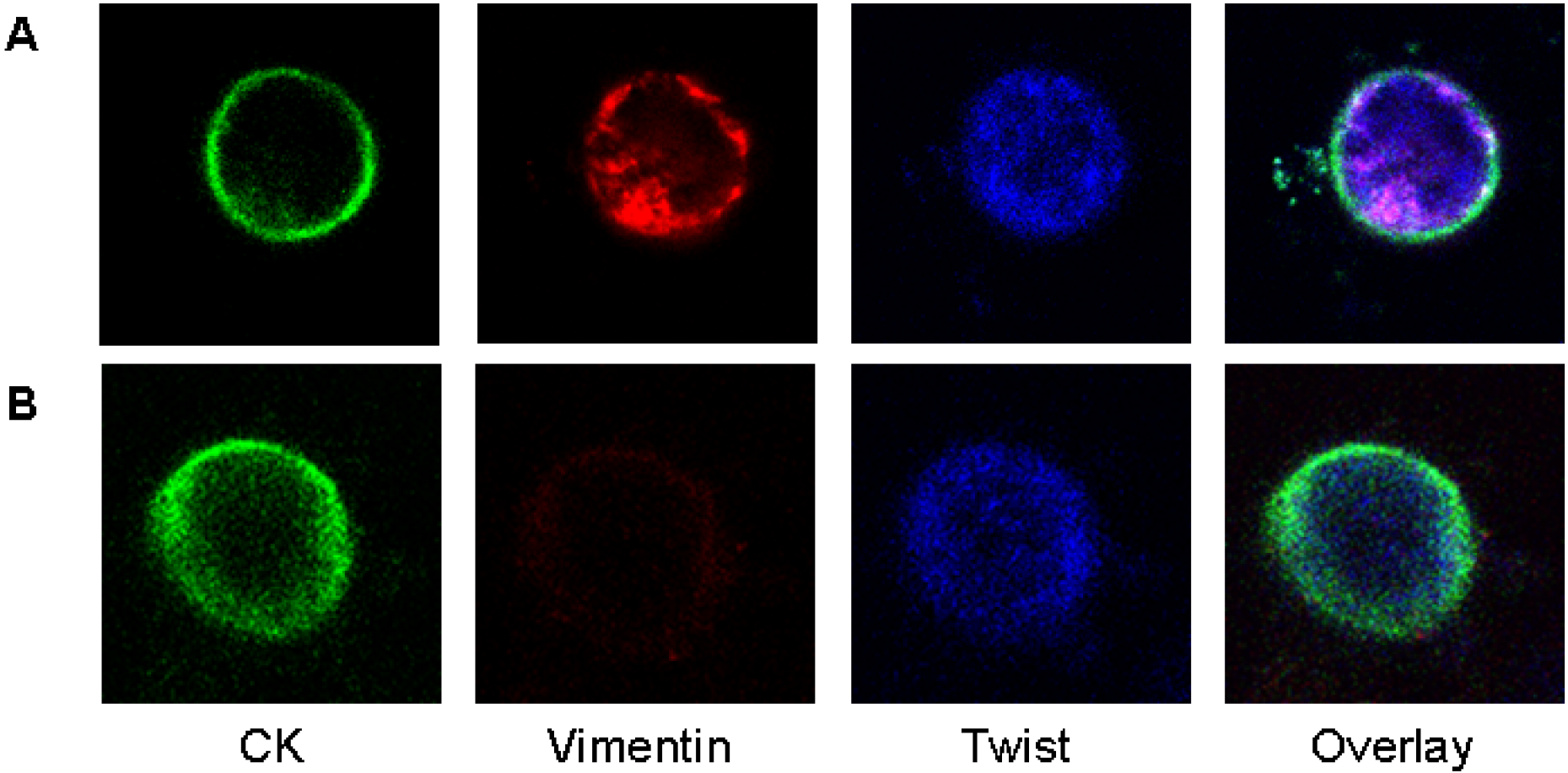

Figure 4

Additional files provided with this submission:

Additional file 1: Supplementary Figure 1.tif, 4221K
http://breast-cancer-research.com/imedia/8832981235433757/supp1.tiff
Additional file 2: Supplementary Figure 2.tif, 2633K
http://breast-cancer-research.com/imedia/3499330754337686/supp2.tiff